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Inhibition of Herpes Simplex Virus Reactivation by Dipyridamole in a Mouse Model

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Herpes simplex virus (HSV) thymidine kinase (TK) has been demonstrated to be important for reactivation from latency. Specifically, HSV latency-associated transcripts (LAT) are expressed during latent infection established by TK-negative (TK^-) HSV mutants, but reactivation is minimal. TK^- HSV, however, readily reactivated in the presence of exogenous thymidine (TdR) in explant medium [Tenser et al. (1996): Journal of Virology 70:1271–1276]. In the present report this was further studied by evaluating the effect of dipyridamole (DPM) on HSV reactivation. DPM is known to interfere with nucleoside transport. Inhibition of TdR-enhanced reactivation of TK^- HSV and inhibition of reactivation of wild-type TK^+ HSV were evaluated in an experimental mouse model of latency. Without DPM, TK^- HSV reactivation was increased from 0% to 88% with TdR in explant medium, demonstrating TdR-enhanced reactivation of TK^- HSV (as seen previously). TdR-enhanced reactivation of TK^- HSV was decreased when DPM (25 or 50 μM) was also present, to 30%–60% and to 0%, respectively. Secondly, DPM also decreased reactivation of wild-type TK^+ HSV. The reactivation frequency of latently infected dorsal root ganglia was 90% in standard medium (no added TdR), and this was decreased by DPM to 9% and 0%, respectively. Reactivation of trigeminal ganglia in standard medium was 100%, and this decreased to 59% and 23%, respectively. The possibility of a direct toxic effect of DPM on ganglion neurons to explain the results was unlikely. DPM had a modest antiviral effect on HSV replication in cell culture, and its efficacy in blocking reactivation may be related to this activity, probably by inhibition of nucleoside transport.

KEY WORDS: HSV latency, nucleotide pools, nucleoside transport, thymidine kinase

INTRODUCTION

Herpes simplex virus (HSV) is a frequent cause of latent infection of human sensory ganglion neurons, and reactivation from latency is the basis of recurrent mucocutaneous infection. Latent infection is readily established in experimental animals, and characteristics of HSV latency have been experimentally defined [Ho, 1992; Stevens, 1989; Tenser, 1994]. During latency, HSV antigens are not expressed and a single species of viral RNA, the latency-associated transcript (LAT), is readily detected. In experimental studies, by the use of HSV mutants, it has been possible to investigate the role of viral functions in HSV latency. For example, by the use of replication-defective HSV mutants, it has been reported that in vivo HSV replication is not necessary for the establishment of latency [Leib et al., 1989; Sedarati et al., 1993; Steiner et al., 1990]. In studies with HSV mutants defective for viral thymidine kinase (TK) expression, it has been reported that viral TK expression is important for latency. Specifically, since LAT is expressed during latent infection established by TK negative (TK^-) HSV mutants, but reactivation from latency is impaired, it was concluded that HSV TK expression is important for reactivation from latency [Coen et al., 1989; Efstathiou et al., 1989; Leist et al., 1989; Tenser, 1994; Tenser et al., 1989].

In initial biological studies of HSV TK, it was suggested that HSV TK expression was important for replication in nondividing cells, but was dispensable for replication in dividing cells [Jamieson et al., 1974]. Since neurons, the site of HSV latency, are nondividing, it was suggested that HSV TK expression might be important for infection of these cells. This has generally been supported in studies of reactivation of TK^- HSV, as well as in the study of acute ganglion infection by TK^- HSV [Tenser, 1994]. The mechanisms which result in impaired TK^- HSV reactivation and replication in neurons are uncertain, but may be due to suboptimal nucleotide pools [Jacobson et al., 1995], possibly secondary to low levels of cellular cytosolic TK (TK 1). Low neuronal TK

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levels would likely result in thymidine (TdR) nucleotide pools in neurons which are suboptimal for replication of HSV, particularly TK-deficient HSV. With this scenario in mind, and in order to attempt to increase cellular TdR nucleotide pools, we investigated whether reactivation of TK^- HSV from latency might be enhanced if ganglia latently infected with TK^- HSV were reactivated in the presence of increased TdR. Markedly increased reactivation of TK^- HSV was observed; reactivation rates increased from approximately 0% to approximately 90% [Tenser et al., 1996].

In the present study, the mechanism by which TdR might enhance TK^- HSV reactivation was investigated by the use of dipyridamole (DPM), an inhibitor of nucleoside transport [Bannister, 1992; Fitzgerald, 1987; Patel et al., 1991; Plagemann and Wohlheuter, 1985; Westley and Schaefer, 1994]. It was thought that if transport of added exogenous TdR into neurons was necessary for reactivation of TK^- HSV, DPM would inhibit TdR-enhanced reactivation of TK^- HSV. This was observed, suggesting that if neuronal TdR was increased, TK^- HSV would be reactivation competent. In addition, the effect of DPM on reactivation of wild-type TK^+ HSV was investigated. DPM inhibited wild-type HSV reactivation in a dose-dependent manner, to a greater degree in dorsal root ganglia (drg) than in trigeminal ganglia (tg). The effect of DPM on TK^- and TK^+ HSV reactivation suggested that TdR transport and presumably intracellular nucleotide pool levels can be altered with exogenous TdR or with inhibitors of nucleoside transport, and that altered HSV reactivation may result. It is hypothesized that even a modestly effective antiviral such as DPM may be effective in blocking reactivation, since during the process of HSV reactivation the viral burden is small.

MATERIALS AND METHODS

Viruses

Standard TK^+ HSV (HSV-1 strain KOS) and the dlsactk deletion mutant derived from the KOS strain were used [Coen et al., 1989; Tenser et al., 1996]. Viruses were grown and titrated in Vero cells by standard methods. Stock titers were 10^8 PFU/ml for the TK^+ virus and 4×10^8 PFU/ml for the TK^- virus. TK activity of the mutant TK^- virus was less than 1% of the TK^+ virus, as determined by in vitro thymidine phosphorylation [Tenser et al., 1996].

Animals and Virus Inoculation

Latent HSV infection of drg and tg was established in randomly bred CD-1 mice (Charles River Laboratories, Wilmington, MA) by previously described methods [Tenser et al., 1996]. In brief, under general anesthesia (methoxyflurane) bilateral footpad or bilateral corneal inoculation was performed using stock virus containing 10^7 PFU/ml (25 μl per footpad). After 4–6 weeks, drg were considered to be latently infected and were assayed for HSV reactivation in explant culture.

Explant Reactivation

During the period of latency, drg and tg were removed from anesthetized mice (methoxyflurane), and individual ganglia were assayed for evidence of latency by detection of HSV reactivation. The fourth lumbar (L4) and L5 ganglia from the right and left side and both tg were assayed individually. Six days after explant culture, ganglia were homogenized and HSV was isolated on Vero cells to determine evidence of reactivation. Explant culture medium consisted of medium 199 containing 2% dialyzed calf serum (Gibco BRL, Gaithersburg, MD) using methods as described previously [Tenser et al., 1996]. Culture medium of some ganglia latently infected with TK^- HSV was supplemented with thymidine (Sigma, St. Louis, MO). In some instances, DPM (Sigma) was added to the explant medium.

Growth Curve

Antiviral activity of DPM was tested in HSV growth curves, performed by standard means. In brief, confluent Vero cells were infected with 10^4 PFU of TK^+ HSV (MOI 0.01). After incubation at 37°C for 1 hr, monolayers were washed with cell culture medium and then incubated at 37°C. At varying time points, medium was removed and after monolayers were lysed, cell-free virus was determined by plaque assay. Results are the average of two independent experiments.

Immunoperoxidase

Ganglia were tested for neuron-specific enolase by peroxidase-antiperoxidase methodology. Antibody to neuron-specific enolase (Polysciences, Inc., Warrington, PA) was used at a dilution of 1:1,000 and anti-rabbit IgG and peroxidase-antiperoxidase at dilutions of 1:100 were used. Explanted ganglia were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde. Paraffin sections were prepared by standard means and then assayed. Normal ganglion tissue from an uninfected mouse served as a positive control for the immunoassay.

RESULTS

Although TK^- HSV reactivated poorly, if at all, in explant cultures of latently infected sensory ganglia, addition of TdR to the explant medium greatly enhanced reactivation. As seen in Table I, reactivation of latently infected drg cultivated in standard medium did not reactivate (0%, zero of eight ganglia), but when ganglia were cultivated in medium supplemented with 100 or 200 μM TdR, reactivation rates were 88% (7 of eight ganglia). The TdR effect was similar to that which was observed previously [Tenser et al., 1996]. When DPM was added to the explant medium in addition to TdR, TdR-enhanced reactivation of TK^- HSV was greatly decreased. With 50 μM DPM, the reactivation rate was 0% for cultures explanted in 100 μM TdR (0 of eight ganglia) or 200 μM TdR (0 of eight ganglia). Reactivation in explant cultures containing 25 μM DPM as intermediate (Table I).

Since DPM inhibited TdR-enhanced reactivation of TK^- HSV (Table I), possibly via the inhibition of TdR

TABLE I. Inhibition by DPM of TdR-Enhanced Reactivation of TK⁻ HSV From drg*

TdR supplementation of explant medium (μM)[a]	Ganglia which reactivated HSV/no. tested (%) Concentration of DPM in explant medium (μM)		
	0	25	50
0	0/8 (0)	ND[b]	ND
100	7/8 (88)	3/8 (38)	0/8 (0)
200	7/8 (88)	5/8 (62)	0/8 (0)

*Ganglia latently infected with TK⁻ HSV (4–6 weeks postinfection) were explanted in medium with or without TdR supplementation and with or without DPM. Six days after explant culture, explants were homogenized and tested for HSV.
[a]Exogenous TdR added to explant medium is indicated. Standard explant medium was Medium 199 containing antibiotics and supplemented only with dialyzed bovine serum in an attempt to minimize TdR present. Medium indicated as being 200 μM actually contained TdR, 264 μM, when analyzed by HPLC (methods not shown).
[b]ND, not done.

TABLE II. Inhibition by DPM of TK⁺ HSV Reactivation From Latently Infected Sensory Ganglia

Ganglia tested	Ganglia which reactivated HSV/no. tested (%)[a] Concentration of DPM in explant medium (μM)		
	0	25	50
drg	9/10 (90)	2/22 (9)	0/22 (0)
tg	10/10 (100)	13/22 (59)	5/22 (23)

[a]Ganglia latently infected with TK⁺ HSV (4–6 weeks postinfection) were explanted in medium with or without DPM. Six days after explant culture, explants were homogenized and tested for HSV.

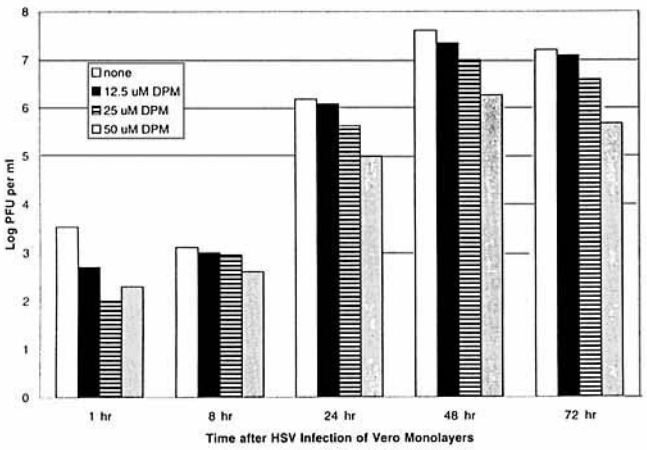

Fig. 1. The effect of DPM on HSV replication in cell culture. Average results of two independent experiments are shown. A modest inhibitory effect with increasing amounts of DPM is apparent.

transport into cells, we investigated inhibition of reactivation of wild-type TK⁺ HSV latent infection. Ganglia cultivated in standard explant medium reactivated at high frequency (90%–100%) as would be expected (Table II). Reactivation was markedly inhibited when explant medium contained DPM, although the effect was greater for drg than for tg. The explanation for the drg-tg difference is unclear. It is noted that we observed a marked difference in the effect of TdR on TK⁻ HSV reactivation from drg and tg [Tenser et al., 1996], and other investigators also noted differences in HSV reactivation from drg and tg [Sawtell and Thompson, 1992; Spivack et al., 1995]. Reactivation frequencies of drg latently infected with TK⁺ HSV in the presence of 25 and 50 μM DPM were 9% (2 of 22 ganglion) and 0% (0 of 22 ganglia), respectively. For tg reactivation, frequencies were 59% (13 of 22 ganglia) and 23% (5 of 22 ganglia), respectively (Table II). Since DPM is known to inhibit nucleoside transport [Patel et al., 1991; Plagemann and Wohlheuter, 1985; Tenser et al., 1996; Westley and Schaefer, 1994] and also inhibited TdR-enhanced reactivation of TK⁻ HSV (Table I), inhibition of wild-type TK⁺ HSV by the inhibition of nucleoside transport was plausible.

It was thought the inhibition of HSV reactivation by DPM might be related to the inhibition of HSV replication, and this was investigated by determination of the effect of DPM on HSV growth curves. As seen in Figure 1, DPM had a modest inhibitory effect on TK⁺ HSV replication. It is hypothesized that DPM inhibited HSV reactivation in latently infected explants by mechanisms similar to the inhibition of HSV in growth curves, possibly due to the inhibition of nucleoside transport. Although only a modest antiviral, DPM may have effectively inhibited reactivation because during initial HSV reactivation events only a very small amount of virus is probably present.

It remained possible that the inhibitory effect of DPM on HSV reactivation was related to damage of latently infected neurons in explants. This possibility is difficult to completely exclude, since multiple factors and assays of neurons might have to be considered. It was investigated in part by immunohistochemical evaluation of neurons for expression of a neuronal antigen and by the evaluation of removing DPM after explants were exposed to the drug for several days. Abundant immunohisto-

TABLE III. Inhibition by DPM of TK+ HSV Reactivation: Decrease of the Effect by Removal of DPM*

	Reactivation of HSV, no. ganglia positive/total (%)		
Ganglia tested	No DPM 6 days	DPM 6 days	DPM 3 days, then no DPM 3 days
drg	9/10 (90)	1/10 (10)	10/20 (50)
tg	10/10 (100)	3/10 (30)	11/20 (55)

*Ganglia latently infected with wild-type TK+ HSV were explanted in standard medium with or without DPM. Concentration of DPM was 25 μM (drg) or 50 μM (tg). In some cultures, DPM was removed after 3 days and explant culture continued for an additional 3 days in standard medium. After 6 days in culture, all explants were homogenized and assayed for HSV.

chemical label was seen with fresh ganglion tissue. Decreased but similar amounts of neuron-specific enolase immunohistochemical label were seen for neurons in explants cultured in medium without or with DPM in the medium (not shown). It was concluded that explant culture of ganglia with or without DPM present resulted in significant neuronal modification. This is probably the result of the many metabolic alterations of the axon reaction [Noguchi et al., 1993; Wong and Oblinger, 1993] coupled with possibly less than optimal conditions for neuronal maintenance in the explant medium used.

To further minimize the possibility that DPM results were due to a toxic effect on ganglion neurons, explants were treated with DPM, and then DPM was removed. Following additional time in culture, explants were assayed for HSV reactivation. It was thought that if HSV reactivation occurred, a DPM neurotoxic effect as a means of explaining DPM-induced inhibition of reactivation would be unlikely. Ganglion explants were treated for 3 days with DPM and then 3 days with standard medium. Reactivation of TK+ HSV occurred in 50% of drg (25 μM DPM) and 55% of tg (50 μM DPM; Table III). These reactivation rates were almost midway between those obtained when DPM was not present at all and when it was present for the entire 6-day period. These results and immunohistochemistry results suggested that the inhibitory effect of DPM on HSV reactivation was not due to neuron destruction by DPM.

DISCUSSION

Although TK- HSV establishes latent infection of sensory ganglion neurons (i.e., LAT is expressed), reactivation is defective. For this reason it has been thought that HSV TK expression is important for the reactivation process [Coen et al., 1989; Efstathiou et al., 1989; Leist et al., 1989; Tenser, 1994; Tenser et al., 1989]. This hypothesis would be in keeping with the early observation that HSV TK expression was important for HSV infection of nonreplicating cells [Jamieson et al., 1974], since sensory ganglion neurons are nonreplicating. It has been suggested that neuronal nucleotide pools are suboptimal for TK- HSV replication and that this can be corrected by HSV TK expression [Jacobson et al., 1995] (i.e., TK+ HSV) or possibly by TdR supplementation [Tenser et al., 1996].

In mammalian cells, cytosolic TK (TK1) and mitochondrial TK (TK2) are expressed, with activity of the former correlating with cell replication [Gross et al., 1987; Hengstschläger et al., 1994; Lieberman et al., 1988; Xu and Plunkett, 1993]. For this reason TK activity has been utilized as a potential marker of neoplastic activity [Hengstschläger et al., 1994; Rehn et al., 1995; Xu and Plunkett, 1993]. Extensive investigations of multiple cell types have demonstrated transcriptional and translational control of TK [Gross et al., 1987; Hengstschläger et al., 1994; Lieberman et al., 1988; Xu and Plunkett, 1993]. Other recent studies have reported on the importance of cellular TK in phosphorylation of antivirals such as AZT to the active triphosphate state [Coen, 1992] and, interestingly, on the competitive inhibition of AZT phosphorylation by TdR [Betageri et al., 1990; Furman et al., 1986]. In neural tissue little TK1 is expressed, and TK2 is the predominant enzyme expressed [Arnér et al., 1992; Gross et al., 1987; Jansson et al., 1992]. Less information is available about the TK activity of neurons present in neural tissue, such as in neurons in sensory ganglia. While TK activity may be expressed in neuronal tumor cells (e.g., neuroblastoma), little if any enzyme activity is present in normal neurons [Rubinstein and Price, 1983; Verri et al., 1992]. Minimal neuronal TK in normal adult neurons, including explant culture, is suggested by the well-recognized lack of TdR uptake by neurons. From this and other information it would be expected that sensory ganglion neuron TK activity would be minimal. In addition, although TMP may be synthesized from dUMP via the thymidylate synthase pathway, this mechanism is not likely to enhance neuronal nucleotide pools. While thymidylate synthase may be detected in neuronal tumor cells [Verri et al., 1992], it is not likely to be found in normal neurons. These factors may result in intraneuronal TdR nucleotide pools that are suboptimal for reactivation of HSV which is defective in viral TK activity.

An additional factor of importance when considering neuronal nucleotide pools is the transport of nucleosides. Several transport mechanisms have been well described [Belt, 1983; Belt et al., 1993; Betageri et al., 1990; Crawford et al., 1990; Plagemann and Wohlheuter, 1985; Plagemann et al., 1988]. Naturally occurring nucleosides diffuse poorly across cell membranes because of lipophilicity [Betageri et al., 1990; Plagemann et al., 1988], and specific transport is necessary. Aside from transport in some specialized cells [Belt et al., 1993; Plagemann et

al., 1988], nucleoside transport is largely of the type noted to be sensitive to nitrobenzylthioinosine (NBTI) and DPM [Belt, 1983; Belt et al., 1993; Betageri et al., 1990; Crawford et al., 1990; Plagemann and Wohlheuter, 1985; Plagemann et al., 1988]. This pathway has been noted to transport multiple nucleosides, including TdR, inosine, guanosine, adenosine, uridine, and cytidine [Belt, 1983; Plagemann et al., 1988]. The NBTI-DPM-sensitive pathway is present in most cell types and is likely to be present in neurons.

In our initial investigation of TdR-enhanced reactivation of TK$^-$ HSV it was thought that increased TK$^-$ HSV reactivation might occur if neuronal TdR nucleotide pools could be improved. It was further thought that this might result from increased TdR in the reactivation medium. Reactivation of TK$^-$ HSV was markedly increased in the presence of increased TdR [Tenser et al., 1996] (Table I), supporting but not proving the hypothesis. The hypothesis was further evaluated in the present study, where DPM, a known inhibitor of nucleoside transport [Bannister, 1992; Fitzgerald, 1987; Patel et al., 1991; Plagemann and Wohlheuter, 1985; Westley and Schaefer, 1994], was used to inhibit TdR-enhanced reactivation of TK$^-$ HSV. This provided additional support for the hypothesis that exogenous TdR resulted in improved intraneuronal nucleotide pools, which resulted in increased TK$^-$ HSV reactivation. Direct measurement of nucleotide pools in ganglia by high performance liquid chromatography (HPLC) is possible, However, results of ganglion assay would indicate total ganglion rather than neuronal levels. Mouse L5 drg contain approximately 4,500 neurons [Tenser et al., 1993] and this is estimated to be no more than 10% of the total ganglion cell population.

Considering the effect of TdR on TK$^-$ HSV reactivation, it seemed possible that 1) TdR had an effect on initial reactivation events or 2) on subsequent replication. After acute in vivo infection of mice, TdR enhanced TK$^-$ HSV replication in ganglia [Tenser et al., 1996]. Based on this observation of enhanced TK$^-$ HSV replication in tg, where it is otherwise restricted, it was thought that the TdR-enhanced reactivation of TK$^-$ HSV may indicate enhanced replication subsequent to initial reactivation events.

In the present study, DPM inhibited TdR-enhanced TK$^-$ HSV reactivation and also inhibited wild-type TK$^+$ HSV reactivation. Previously, a slight antiviral effect of DPM was suggested when it was combined with antiviral drugs [Snoeck et al., 1994]. It is suggested that DPM, known to inhibit nucleoside transport [Bannister, 1992; Fitzgerald 1987; Patel et al., 1991; Plagemann and Wohlheuter, 1985; Westley and Schaefer, 1994], inhibited TdR-enhanced TK$^-$ HSV reactivation by inhibition of TdR transport. By analogy with TK$^-$ HSV results, TK$^+$ HSV inhibition may also have been related to inhibition of nucleoside transport. Inhibition of TdR-enhanced reactivation of TK$^-$ HSV by DPM supported the conclusion that limited TdR nucleotide pools are limiting for reactivation of TK$^-$ HSV [Jacobson et al., 1995; Tenser et al., 1996]. Furthermore, the decrease of TK$^+$ HSV reactivation by DPM suggested that inhibition of nucleoside transport, probably resulting in altered nucleotide pools, may inhibit wild-type HSV reactivation as well. It did not appear that the DPM effect was due to the destruction of latently infected neurons in ganglion explants, an important consideration. This conclusion was supported by evidence that removal of DPM improved reactivation, albeit not to the level of explants without exposure to DPM (Table III). However, explant culture of ganglia results in the apparent disruption of many neurons, and it would not be surprising that the rate of reactivation would be decreased as part of this process. That is, it is probable that some latently infected neurons prevented from reactivation of HSV by the presence of DPM were damaged by the explant culture process, and were not capable of undergoing reactivation when DPM was removed. In addition, while neuron-specific enolase immunoactivity was decreased after DPM treatment, the decrease was no greater than after explant culture without DPM. This suggests no specific DPM neurotoxicity. Lastly, DPM is approved for clinical use as an antiplatelet medication in the treatment of vascular disease [Bannister, 1992; Fitzgerald, 1987; Westley and Schaefer, 1994] and has not been shown to be neurotoxic.

It is suggested that in the present study DPM was of more than modest efficacy in blocking reactivation because of the nature of HSV latency and reactivation. It is likely that during reactivation only minimal amounts of HSV are initially present, and the reactivating viral genome may be in a particularly vulnerable state. Possibly for these reasons even a modestly effective antiviral may be useful.

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